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The interactions of 9,10-phenanthrenequinone with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a potential site for toxic actions

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Abstract

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate, one of the precursors for glycolytic ATP biosynthesis. The enzyme contains an active site cysteine thiolate, which is critical for its catalytic function. As part of a continuing study of the interactions of quinones with biological systems, we have examined the susceptibility of GAPDH to inactivation by 9,10-phenanthrenequinone (9,10-PQ). In a previous study of quinone toxicity, this quinone, whose actions have been exclusively attributed to reactive oxygen species (ROS) generation, caused a reduction in the glycolytic activity of GAPDH under aerobic and anaerobic conditions, indicating indirect and possible direct actions on this enzyme. In this study, the effects of 9,10-PQ on GAPDH were examined in detail under aerobic and anaerobic conditions so that the role of oxygen could be distinguished from the direct effects of the quinone. The results indicate that, in the presence of the reducing agent DTT, GAPDH inhibition by 9,10-PQ under aerobic conditions was mostly indirect and comparable to the direct actions of exogenously-added H_2O_2 on this enzyme. GAPDH was also inhibited by 9,10-PQ anaerobically, but in a somewhat more complex manner. This quinone, which is not considered an electrophile, inhibited GAPDH in a time-dependent manner, consistent with irreversible modification and comparable to the electrophilic actions of 1,4-benzoquinone (1,4-BQ). Analysis of the anaerobic inactivation kinetics for the two quinones revealed comparable inactivation rate constants (k_{inac}), but a much lower inhibitor binding constant (K_i) for 1,4-BQ. Protection and thiol titration studies suggest that these quinones bind to the NAD+ binding site and modify the catalytic thiol from this

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site. Thus, 9,10-PQ inhibits GAPDH by two distinct mechanisms: through ROS generation that results in the oxidation of GAPDH thiols, and by an oxygen-independent mechanism that results in the modification of GAPDH catalytic thiols.

Keywords: GAPDH; 9,10-Phenanthrenequinone; 1,4-Benzoquinone; Hydrogen peroxide

1. Introduction

Quinones are an ubiquitous class of organic compounds of substantial toxicological interest [1–3]. Several potential deleterious quinines, such as 9,10-phenanthrenequinone (9,10-PQ) have been found in the particulate fractions of air pollution samples including ambient air and diesel exhaust particles [4,5]. Such quinones are of toxicological interest because these particles exhibit toxicity that is consistent with quinone-like chemistry [6,7,3,8].

The toxicity of quinones has been proposed to be the result of at least two mechanisms: (I) their direct actions as electrophiles, leading to covalent modification of nucleophilic functions, such as thiols on vital cellular components, and (II) their ability to act as catalysts in the generation of reactive oxygen species (ROS) [1,9]. As substrates for cellular reductases (e.g., P450 reductase), quinones can undergo one-electron reduction to yield semiquinone radical anions, which can potentially reduce molecular oxygen, resulting in the catalytic production of superoxide (O_2^-). Thus, a single quinone molecule has the potential of catalytically generating O_2^-, which can dismutate to hydrogen peroxide and oxygen spontaneously or by superoxide dismutase (SOD) catalysis [1,9,10]. In this way, quinone-mediated generation of O_2^- and H_2O_2 can lead to cellular oxidative stress, ultimately ending in cellular demise.

In a study of quinone toxicity in yeast, we found 9,10-phenanthrenequinone, whose actions were thought to be indirect and primarily based on oxygen reduction, to be toxic under anaerobic conditions [11]. In that study, 9,10-PQ induced cell viability loss under anaerobic conditions with an IC_{50} value of approximately 12.0 μM. This effect positively correlated with a reduction in the glycolytic activity of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), suggesting possible direct actions by this quinone on this enzyme.

GAPDH catalyzes the oxidative phosphorylation of glyceraldehyde-3-phosphate (G-3-P) to 1,3-diphosphoglycerate (1,3-DPG), an intermediate for glycolytic ATP synthesis. The postulated catalytic mechanism involves an active site cysteine thiolate, which reacts with the aldehyde group of G-3-P to form a thiohemiacetal intermediate (I), which upon a hydride transfer to a tightly bound nicotinamide cofactor (NAD+), collapses to an acyl thioester (II). After dissociation of

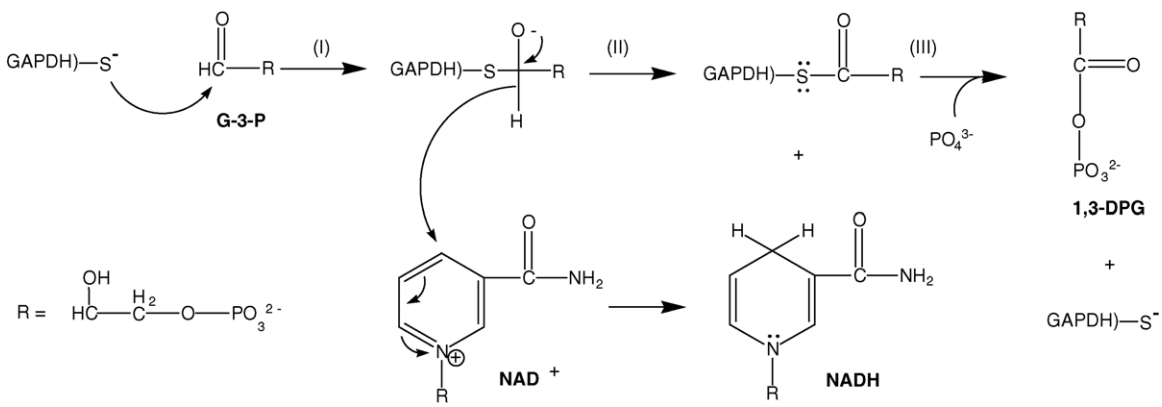

Fig. 1. The proposed catalytic mechanism for GAPDH. (I) An active site thiolate attacks the aldehyde group of G-3-P to generate a thiohemiacetal intermediate. (II) Upon a hydride transfer to NAD+, a thioester is generated which is subsequently displaced by inorganic phosphate (III) to generate 1,3-diphosphoglycerate (1,3-DPG).

NADH and binding of another NAD+ equivalent, inorganic phosphate attacks and displaces the acyl thioester to generate 1,3-DPG (III) (Fig. 1) [12–14].

The active site cysteine thiolate of GAPDH has been reported to be highly reactive and susceptible to alkylation by chemically-diverse electrophiles, such as iodoacetic acid [12], acrylonitrile [15], *N*-acetyl-*p*-benzoquinone imine [16] and vinyl sulfones [17]. This catalytically-active sulfhydryl group is also readily susceptible to oxidation by ROS, such as H_2O_2 [18–20]. In all cases, sulfhydryl modification results in enzyme inactivation which blocks glycolytic ATP synthesis and may also disrupt the numerous other functions associated with GAPDH (e.g., DNA replication and repair) [17,21,19].

This report describes the results of a study detailing the interactions of 9,10-PQ with GAPDH under aerobic and anaerobic conditions so that the role of oxygen could be distinguished from the direct effect(s) of the quinone. These actions were compared with those of 1,4-BQ, whose actions are primarily electrophilic and independent of oxygen, and hydrogen peroxide, whose generation is presumably catalyzed by 9,10-PQ under aerobic conditions. The results indicate that 9,10-PQ inhibits GAPDH by two distinct mechanisms.

2. Experimental

2.1. Materials

9,10-Phenanthrenequinone, 1,4-benzoquinone (1,4-BQ), and 5,5′-dithiobis(2-nitrobenzoic acid) were purchased from Aldrich (Milwaukee, WI). Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) from the yeast *Saccharomyces cerevisae* was obtained as a lyophilized powder (94–120 units/mg protein) from Sigma (St. Louis, MO). Glyceraldehyde-3-phosphate, β-nicotinamide adenine dinucleotide (NAD^+), reduced glutathione (GSH), hydrogen peroxide (30%, w/w), dithiothreitol (DTT), superoxide dismutase (SOD), and catalase were also purchased from Sigma. Sodium azide was purchased from Matheson, Coleman, and Bell (Norwood, OH). All other chemicals were purchased from commercial suppliers and were of the highest quality available.

GAPDH was dissolved in phosphate buffer (0.1 M, pH 7.4), aliquoted, and stored at −80 °C until ready for use.

The quinones were dissolved in acetone (HPLC grade) to give a 10 mM stock solution. Serial dilutions were then performed to achieve the desired concentrations. The final concentration of acetone in the incubation mixtures was 3.4%.

2.2. Dialyzed GAPDH

GAPDH, as received from Sigma, was first subjected to reductive treatment by dissolving the lyophilized powder (500 units) in 5.0 mL phosphate buffer (0.1 M, pH 7.4) containing 1.0 mM DTT. The mixture was then dialyzed in Slide-A-Lyzer dialysis cassettes (10,000 MWCO, Pierce Rockford, IL) against 1 L of phosphate buffer (0.1 M, pH 7.4) for 20 h under a positive pressure of nitrogen gas (99.997% purity). The dialysis buffer was changed at least four times after being purged with nitrogen gas for at least 20 min. Dialyzed GAPDH was aliquoted and stored at −80 °C until ready for use.

2.3. GAPDH assay

GAPDH activity was assayed by monitoring the formation of NADH at 340 nm, based on a modification of the method of Dagher and Deal [22]. In brief, GAPDH (0.48 units/mL) was assayed in a 1.0 mL volume containing 4.0 mM GSH, 1.0 mM NAD^+, and 1.5 mM G-3-P. NADH formation was monitored every 12 s for 1 min using a double-beam Uvikon spectrophotometer and GAPDH activity expressed as micromoles of NADH formed per minute per milligram of protein, using an extinction coefficient of 6290 M^{-1} cm^{-1}.

2.4. Aerobic incubations

Incubations were carried in opened glass tubes (16 mm × 100 mm) to allow atmospheric oxygen into the headspace. Mixtures consisted of GAPDH (1.0 units/mL), DTT (1.0 mM), and different concentrations of 9,10-PQ in a total volume of 1450 μL. Following the addition of 9,10-PQ, mixtures were incubated in a shaking bath for 60 min at ambient temperature. A volume of 750 μL was then withdrawn and assayed in a 1.0 mL volume containing 1.5 mM G-3-P, 1.0 mM NAD+, and 4.0 mM GSH. For reversibility studies, GAPDH was assayed again following a 20-min incubation with excess DTT (12.5 mM).

Incubations with 1,4-BQ were performed similarly by incubating GAPDH (2.2 units/mL) for 10 min with different concentrations of 1,4-BQ in the presence and absence of DTT (100 μM) in a volume of 150 μL. Following incubation, a 35-μL aliquot was withdrawn and assayed for remaining activity in a 1.0 mL volume containing 1.0 mM NAD+, 1.5 mM G-3-P, and 4.0 mM GSH.

2.5. GAPDH inhibition by hydrogen peroxide

GAPDH (2 units/mL) was incubated with different concentrations of hydrogen peroxide in the presence of GSH (4.0 mM) in a final volume of 150 μL. Following a 1-h incubation, a 35-μL aliquot was assayed for remaining activity in a 1.0 mL volume containing 1.0 mM NAD+, and 0.5 mM G-3-P. The remaining mixture was treated with excess DTT (22.7 mM), and following a 30-min incubation, a 35-μL aliquot was assayed for remaining activity.

2.6. Protection studies with catalase

Aerobic incubations were carried out as described above, except that they included catalase (100 U/mL) and 9,10-PQ (12.50 μM) in a total volume of 725 μL. Incubations with azide were performed by first pre-incubating mixtures containing catalase (100 units/mL) and sodium azide (100 μM) for 5 min before the addition of 9,10-PQ. A 700-μL aliquot was withdrawn to assay for remaining activity.

2.7. Time-dependent inactivation

Incubations were carried out in glass tubes (16 mm × 100 mm) equipped with gas tight rubber septa. Deoxygenation was performed by first purging dialyzed GAPDH (0.69 units/mL) in phosphate buffer (0.1 M, pH 7.4) with nitrogen gas (99.997% purity) for 15–20 min before the addition of quinone via a gas-tight syringe. Incubations were performed in the presence of various concentrations of 9,10-PQ or 1,4-BQ in a total volume of 725 μL for 0, 1, 2, and 5 min. A volume of 700 μL was withdrawn and assayed for residual activity in a 1.0 mL volume containing 1.5 mM G-3-P, 1.0 mM NAD+, and 4.0 mM GSH. Aerobic inactivation rates were determined similarly except no deoxygenation was performed and the incubation mixtures were opened to atmospheric oxygen. Incubations were carried out for 20, 30, and 60 min in the presence of 1.0 mM DTT.

2.8. Protection studies

Dialyzed GAPDH was pre-incubated anaerobically with excess of either G-3-P (1.8 mM) or NAD+ (1.2 mM) for 5 min before the addition of either 9,10-PQ (12.5 μM) or 1,4-BQ (1.0 μM). Following a 30-min incubation, a 700-μL aliquot was withdrawn to assay for residual activity as previously described.

2.9. Titration of GAPDH thiols

Dialyzed GAPDH (0.61 μM) in a volume of 1.4 mL of phosphate buffer (0.1 M, pH 7.4) was deoxygenated for 15–20 min as previously described. Following the addition of different concentrations of quinone via gas-tight syringe, mixtures were incubated under nitrogen for 1.5–2.0 h at room temperature. A 100-μL aliquot was then withdrawn to assay for residual activity and a volume of 1300 μL was subjected to DTNB treatment to determine modifications on GAPDH thiols. For DTNB treatment, the mixture had to be concentrated to increase sensitivity and unreacted quinone had to be removed because of spectroscopic interference. This was achieved using Nanosep® centrifugal devices (30,000 MWCO, Pall Life Sciences, Ann Arbor, MI). In brief, incubation mixtures were centrifuged (14,000 × *g*, 4 °C, 8 min), and the remaining residue was subjected to four wash-centrifuge cycles (14,000 × *g*, 4 °C, 8 min) with a 20% acetone aqueous solution. The final residue was re-suspended in 50 μL tris buffer (0.1 M, pH 8.5) and subjected to denaturing treatment with 2.7 M guanidine hydrochloride (pH 8.5). The final mixture was treated with 0.5 mM DTNB (in methanol) in a final volume of 100 μL. The resulting absorbance at 412 nm was measured in a 50 μL quartz cuvette using a Shimadzu UV–vis recording spectrophotometer.

2.10. Data analysis

The time-dependent inactivation of GAPDH by 9,10-PQ or 1,4-BQ was analyzed according to the method of Kitz and Wilson [23] with minor modifications. In brief, inactivation rate constants,

k_{obs}, were obtained by linear regression analysis (Graphpad Prism, San Diego, CA) of semilog plots of remaining activity (E/E_0) versus time. A good linear correlation was observed for all of the quinone concentrations tested ($r^2 > 0.90$). The inhibitor binding constant, K_i, and the rate constant for the formation of irreversible enzyme–inhibitor complex, k_{inac}, for each quinone were obtained by pooling all of the data for each quinone and plotting the resulting k_{obs} versus quinone concentration and analyzing by non-linear regression for fit to the equation $k_{obs} = k_{inac} \times [quinone]/(K_i + [quinone])$ (Graphpad Prism, San Diego, CA). A good fit was observed for both quinones ($r^2 > 0.90$).

3. Results

3.1. GAPDH inhibition by 9,10-PQ under aerobic conditions

GAPDH has been reported to be highly susceptible to oxidative inactivation by ROS, particularly H_2O_2 [18,24,19,25]. The basis for the inactivation is presumably due to oxidation of the catalytic thiol to sulfenic (SOH), sulfinic (SO_2H), or sulfonic (SO_3H) acids. Sulfenic acids represent unstable intermediates that readily react with either nearby thiols to generate intramolecular disulfides or small thiols, such as glutathione to generate protein mixed disulfides [26]. GAPDH contains two sulfhydryl groups per monomer, which are not in sufficient proximity to form an internal protein disulfide, and thus only protein mixed disulfides have been observed [27,24]. These disulfides are more resistant than sulfenic acids to further oxidation and can be reduced by DTT back to the thiol, thereby regenerating the active protein [28,29,26,30].

Studies by Kumagai et al. [31] indicated that 9,10-PQ efficiently transferred electrons from DTT to oxygen, thereby generating ROS, and Bova et al. [32] have directly measured the DTT-mediated production of H_2O_2 by this quinone. Therefore, as an initial approach to examine the aerobic actions of 9,10-PQ on GAPDH, the enzyme was incubated for 1 h aerobically with different concentrations of 9,10-PQ in the presence of 1.0 mM DTT. As shown in Fig. 2A, 9,10-PQ inhibited GAPDH at concentrations ranging from 0.78 to 6.25 μM under aerobic conditions. Increasing the concentration of 9,10-PQ did not result in much further inhibition under aerobic conditions, consistent with DTT being the limiting reagent in this reaction (data not shown). If the inhibition were due to protein mixed-disulfide formation as a result of oxidation of GAPDH thiols to sulfenic acids followed by thiolation by DTT (Fig. 3), addition of excess DTT should reduce the disulfide back to the thiol and reactivate the enzyme. As indicated by Fig. 2A, the inhibition observed by 9,10-PQ was mostly reversible upon addition of excess DTT. Notably, over 70% of the enzymatic activity was recovered for concentrations of 9,10-PQ (3.12 and 6.25 μM) at which the enzyme was completely inhibited. The incomplete restoration of enzyme activity could be attributed to further oxidation of sulfenic acids to sulfinic and sulfonic acids, which would not be reversible by DTT. In contrast to sulfenic acids, which can be reduced to the thiol by thiol exchange reactions, higher oxidation states, such as sulfinic and sulfonic acids cannot [28,26]. Therefore, oxidation of GAPDH thiols to sulfinic and sulfonic acid states would result in irreversible inhibition of GAPDH, as indicated by Fig. 3.

GAPDH was also inhibited by H_2O_2 in a concentration-dependent manner and, in this case, the inhibition was almost completely reversible by addition of excess DTT, as shown by Fig. 2B. These results are consistent with the oxidation of GAPDH thiols by H_2O_2 to protein mixed disulfides through a sulfenic acid intermediate (Fig. 3). A similar trend of inhibition and reversibility was observed when either GSH or DTT was used in the initial incubation. When incubations were carried out in the absence of these small thiols, the inhibition was not reversible upon addition of DTT, suggesting further oxidation of GAPDH thiols to sulfinic and sulfonic acids by H_2O_2 (data not shown).

When compared to the actions of H_2O_2, 9,10-PQ inactivated GAPDH at much lower concentrations and the inactivation was reversible to a lesser extent than with H_2O_2, possibly due to further oxidation of sulfenic acids by ROS or a different mechanism of inactivation by 9,10-PQ. To further support the involvement of ROS, particularly H_2O_2, in the DTT-mediated aerobic inhibition of GAPDH by 9,10-PQ, incubations were carried out in the presence of catalase. As indicated by Table 1, catalase blocked the aerobic inhibitory effects of 9,10-PQ on GAPDH. The protective effect of catalase was almost completely abolished when incuba-

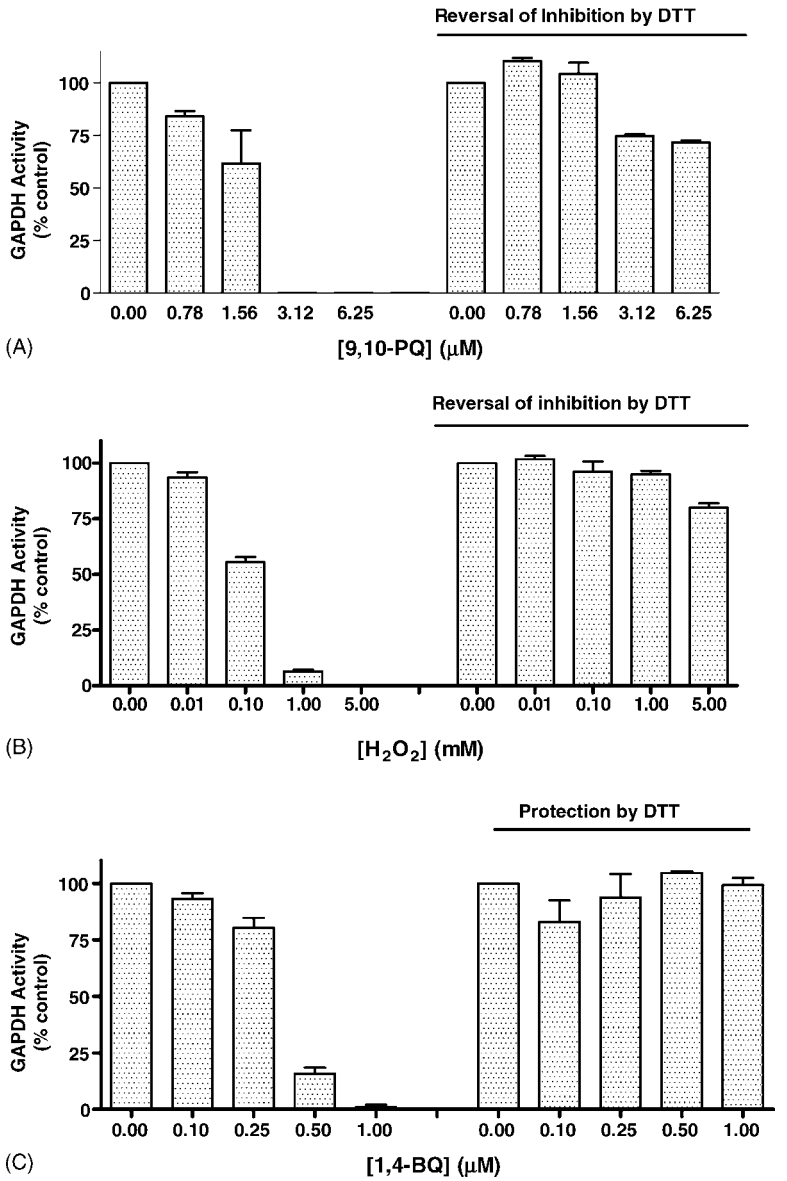

Fig. 2. (A) GAPDH inhibition by 9,10-PQ under aerobic conditions. GAPDH activity was measured following a 1-h aerobic incubation with different concentrations of 9,10-PQ in the presence of DTT (1.0 mM). Reversibility of inhibition was measured following a 20-min incubation with excess DTT (11.7 mM). (B) GAPDH inhibition by hydrogen peroxide. GAPDH activity was measured following a 1-h incubation with different concentrations of H_2O_2 in the presence of 4.0 mM GSH. Reversibility of inhibition was measured after the addition of excess DTT (22.7 mM). (C) GAPDH inhibition by 1,4-BQ under aerobic conditions. GAPDH activity was measured following a 10-min incubation with different concentrations of 1,4-BQ in the presence and absence of DTT (100 μM). In all cases, values represent the average of triplicate determinations ± S.E.

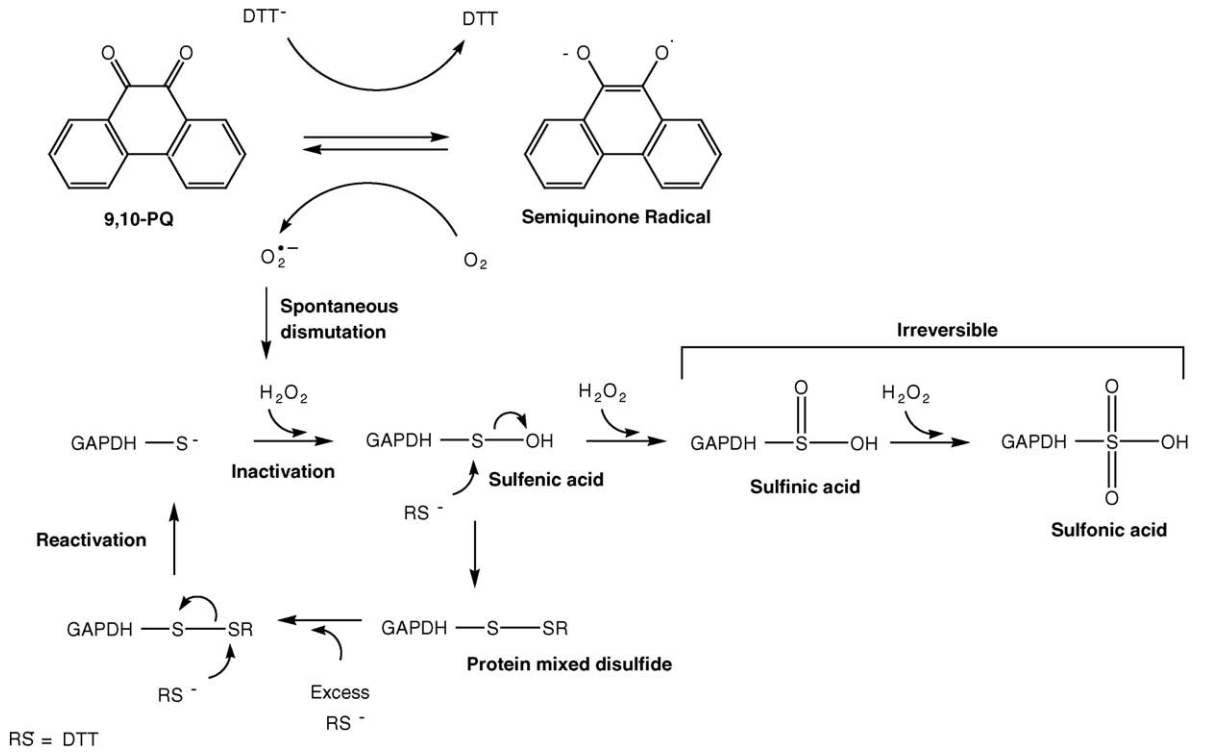

Fig. 3. Proposed mechanism of GAPDH inhibition by 9,10-PQ under aerobic conditions. The one-electron reduction of 9,10-PQ by DTT generates the corresponding semiquinone radical anion, which in turn reduces oxygen to superoxide. The spontaneous dismutation of superoxide generates hydrogen peroxide, which oxidizes GAPDH thiols to sulfenic acids. In the presence of small thiols, sulfenic acids lead to the formation of GAPDH mixed disulfides which can be reduced by the addition of excess thiol, resulting in the reactivation of GAPDH.

tions included 100 μM azide, a potent inhibitor of catalase, supporting the notion that protection is most likely due to the ability of catalase to inactivate H_2O_2. Similar protective effects by catalase have been observed in the generation of H_2O_2 by other quinones [32].

The actions of 9,10-PQ were also compared to the direct actions of 1,4-BQ as an electrophile (Fig. 4). As shown in Fig. 2C, 1,4-BQ inactivated GAPDH under aerobic conditions at concentrations of 0.10–1.0 μM, but unlike 9,10-PQ, the inactivation was completely prevented by high concentrations of DTT. Although DTT exhibited a protective effect on GAPDH, it failed to reverse the effects of 1,4-BQ once the inactivation had occurred. Together these observations are consistent with a direct, electrophilic addition of 1,4-BQ to GAPDH thiols. This DTT effect on the actions of 1,4-

Table 1
Protective effects by catalase on the aerobic inhibition of GAPDH by 9,10-PQ

Experimental conditions	GAPDH activity (% control)
GAPDH + DTT (100 μM)	100.0
GAPDH + DTT (100 μM) + 9,10-PQ	10.54 ± 0.12
GAPDH + DTT (100 μM) + 9,10-PQ + catalase (100 U/mL)	70.37 ± 1.76
GAPDH + DTT (100 μM) + 9,10-PQ + catalase (100 U/mL) + azide (100 μM)	17.64 ± 1.24

GAPDH was incubated aerobically with either catalase (100 U/mL), or catalase (100 U/mL) plus azide (100 μM) for 1-h with 9,10-PQ in the presence of DTT (100 μM). Following incubation, GAPDH activity was measured and the percent inhibition relative to control determined. Values represent the means ± S.E. for triplicate determinations.

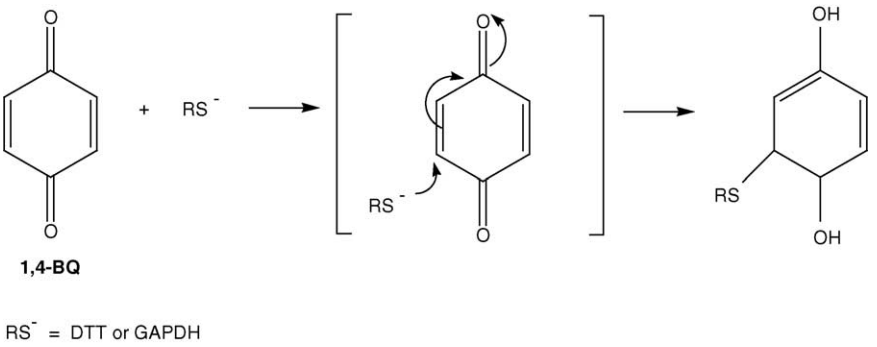

Fig. 4. Electrophilic arylation by 1,4-benzoquinone (1,4-BQ).

BQ contrasts with those of 9,10-PQ and indicates that, under aerobic conditions, 9,10-PQ inactivates GAPDH indirectly through the formation of ROS, particularly H_2O_2 which in turn oxidizes thiols on the enzyme.

3.2. Inactivation of GAPDH by 9,10-PQ under anaerobic conditions

To study oxygen-independent interactions, dialyzed GAPDH was incubated for various times with different concentrations of 9,10-PQ under anaerobic conditions, and as shown in Fig. 5A, a time- and concentration-dependent inactivation of GAPDH was observed. Further examination revealed that this quinone failed to act as a typical reversible competitive inhibitor with respect to either NAD+ or G-3-P, and required pre-incubation with GAPDH to exert its effects (data not shown). For comparison, the anaerobic inactivation of GAPDH by 1,4-BQ as an electrophile was also examined (Fig. 5B), and a similar effect was observed. The kinetic profile of inactivation for each quinone was analyzed according to the method of Kitz and Wilson, and the resulting kinetic parameters are presented in Table 2 [23]. Plots of the observed inactivation rates (k_{obs}) versus quinone concentration exhibited saturation kinetics, consistent with irreversible inhibition (Fig. 5A and B (inset)). The major difference observed between the two quinones was in the K_i, the dissociation constant for the enzyme–inhibitor complex. The calculated values for K_i were 0.67 μM and 22.54 μM for 1,4-BQ and 9,10-PQ, respectively, indicative of a greater affinity for the enzyme by 1,4-BQ. Although 9,10-PQ exhibited a lower affinity for GAPDH, its inactivation rate constant, k_{inac}, of 1.47 $\mu M^{-1}\,min^{-1}$ was comparable to that of 1,4-BQ which was 0.57 $\mu M^{-1}\,min^{-1}$. For comparison, we have also included k_{inac} for the aerobic inactivation process by 9,10-PQ. As also shown in Table 2, the aerobic inactivation process is significantly slower with a k_{inac} of 0.078 $\mu M^{-1}\,min^{-1}$, which may reflect the indirect, ROS generation mechanism of inactivation. Together these observations point to a distinct anaerobic mechanism of GAPDH inactivation by 9,10-PQ.

3.3. The effect of substrate on GAPDH inhibition by 9,10-PQ and 1,4-BQ

In an effort to examine the binding site(s) of these quinones, GAPDH was pre-incubated with excess amounts of either NAD+ or G-3-P before quinone

Table 2
Parameters for the inactivation of GAPDH by 9,10-PQ under aerobic and anaerobic conditions

Quinone	Condition	K_i (μM)	k_{inac} ($\mu M^{-1}\,min^{-1}$)
9,10-PQ	Anaerobic	22.54 ± 13.12	1.47 ± 0.61
9,10-PQ	Aerobic	–	0.078 ± 0.020
1,4-BQ	Anaerobic	0.67 ± 0.22	0.57 ± 0.09

Dialyzed GAPDH was incubated either aerobically or anaerobically with different concentrations of 9,10-PQ for various times, and the resulting enzymatic activity measured. The inhibitor binding constant, K_i, and the rate constant for the formation of irreversible enzyme–inhibitor complex, k_{inac}, were determined by plotting inactivation rates (k_{obs}) vs. quinone concentration (see Fig. 6A and B) and analyzing by non-linear regression for fit to the equation $(k_{obs} = k_{inac} \times [quinone])/(K_i + [quinone])$ (Graphpad Prism, San Diego, CA). Values represent best fit values ± S.E. No K_i value is reported for the aerobic inactivation process by 9,10-PQ due to its indirect nature.

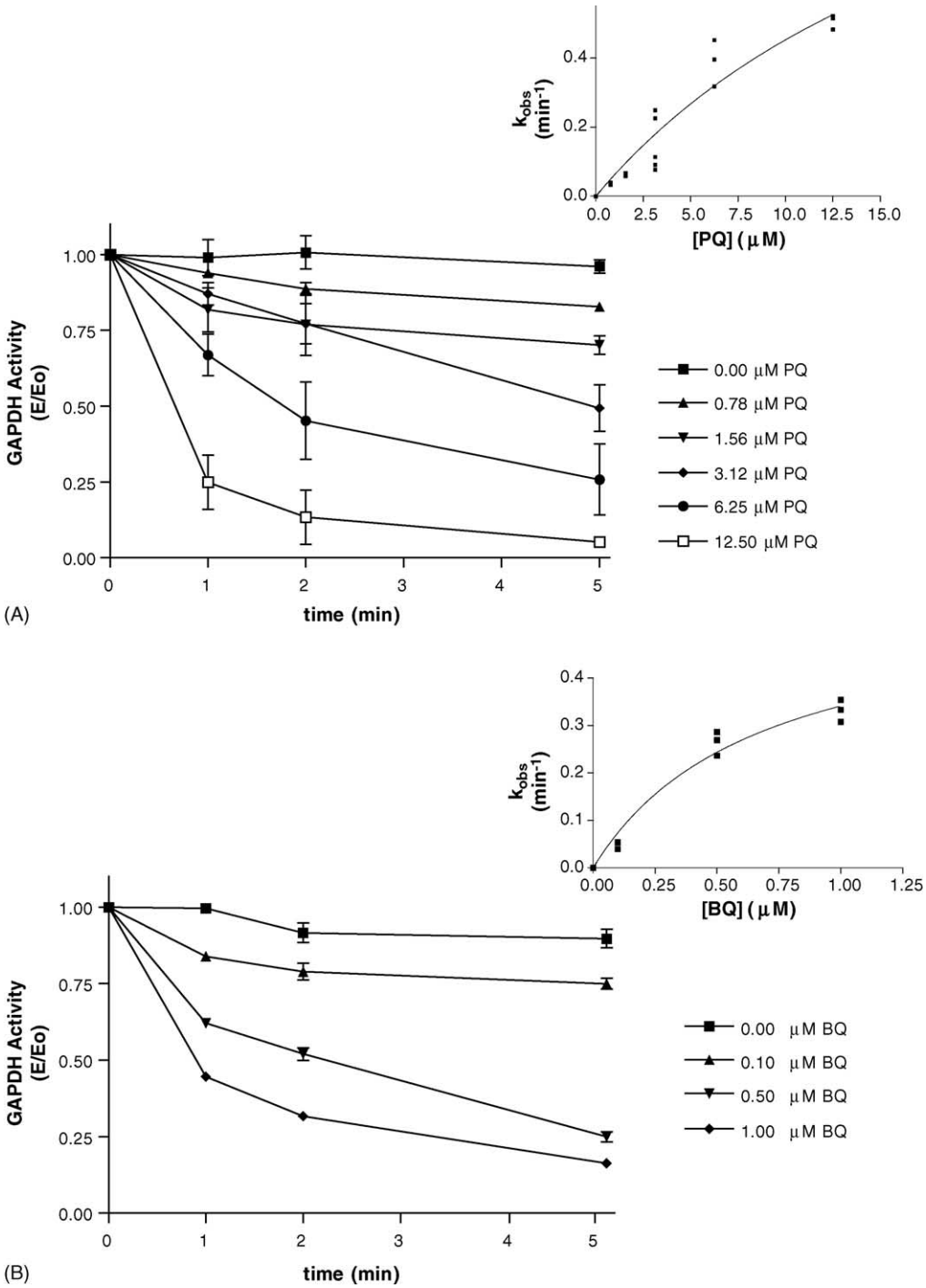

Fig. 5. Time-dependent inactivation of GAPDH by (A) 9,10-PQ and (B) 1,4-BQ under anaerobic conditions. Dialyzed GAPDH was incubated for various times with different concentrations of quinone under anaerobic conditions and the resulting enzymatic activity measured. Values represent the average of triplicate determinations ± S.E. Inset: observed inactivation rates (k_{obs}) are plotted as a function of quinone concentration.

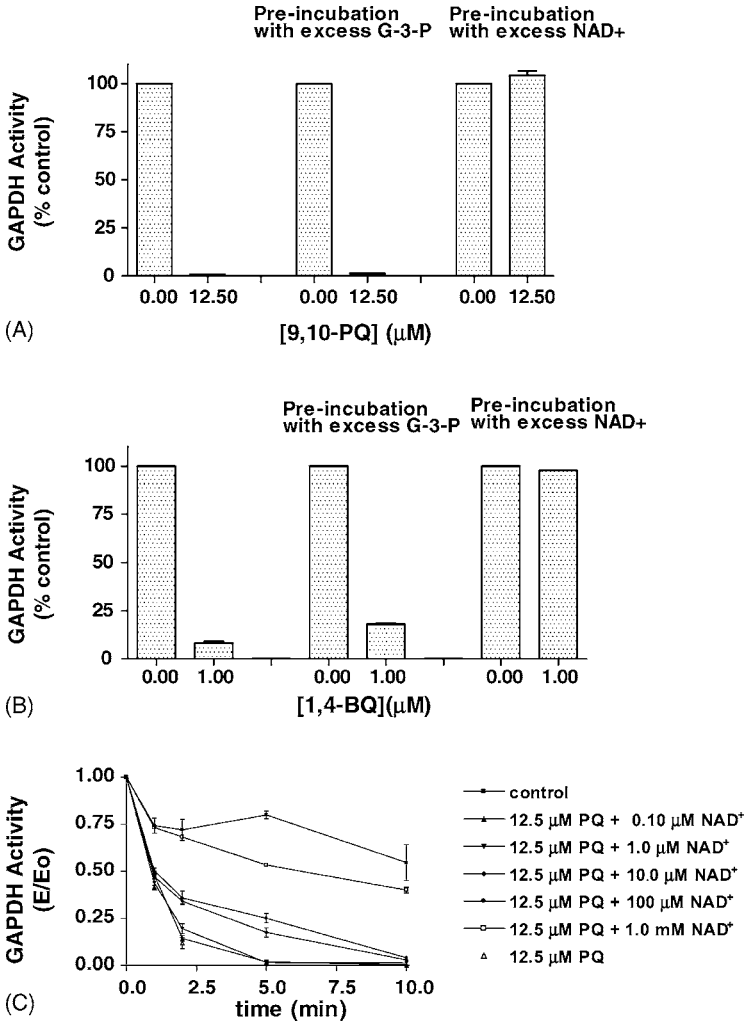

Fig. 6. Protection of GAPDH by NAD+. Dialyzed GAPDH was pre-incubated with excess with either G-3-P or NAD+ for 5 min and then exposed to either (A) 9,10-PQ (12.50 μM) or (B) 1,4-BQ (1.0 μM) for 30 min before measuring enzymatic activity. (C) Concentration-dependent protection by NAD+. GAPDH was pre-incubated with different concentrations of NAD+ for 5 min and then exposed to 9,10-PQ (12.50 μM) for various times. In all cases, values represent averages of triplicate determinations ± S.E.

exposure, and protection against inactivation was assessed. As shown in Fig. 6A and B, NAD+, but not G-3-P, protected GAPDH from inactivation by both 9,10-PQ and 1,4-BQ. Further examination revealed a concentration-dependent protective effect by NAD+ in the inactivation of GAPDH by 9,10-PQ, which was also time-dependent (Fig. 6C). A similar protective effect was observed in the case of 1,4-BQ (data not shown). It should be noted that although NAD+ exhibited protection, it failed to reverse the inactivation of GAPDH by these quinones, which is consistent with the irreversible nature of the inactivation. Thus, 9,10-PQ and 1,4-BQ may exert their actions on GAPDH after binding to the NAD+ binding site.

3.4. Sulfhydryl modification by 9,10-PQ and 1,4-BQ under anaerobic conditions

To further examine the mechanism of GAPDH inactivation by 9,10-PQ under anaerobic conditions,

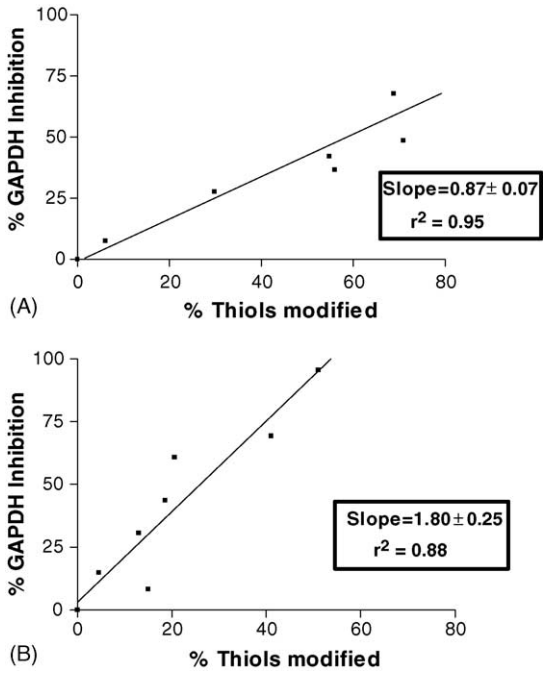

Fig. 7. Modification of GAPDH thiols by (A) 9,10-PQ and (B) 1,4-BQ under anaerobic conditions. Dialyzed GAPDH was incubated for 1.5–2.0 h anaerobically with different concentrations of either (A) 9,10-PQ or (B) 1,4-BQ. Following incubation, an aliquot was used to assay GAPDH activity and the rest was filtered to remove unreacted quinone, treated with the denaturing agent guanidine–HCl (2.7 M, pH 8.5), and analyzed for sulfhydryl modification using DTNB.

the relationship between enzymatic activity and sulfhydryl-modification was assessed following quinone treatment. Thus, dialyzed GAPDH was incubated anaerobically with different concentrations of quinone for 1.5–2.0 h, and following treatment with the denaturing agent guanidine–HCl, GAPDH thiols were titrated with the sulfhydryl-modifying agent 3,3′-dithio-bis(6-nitrobenzoic acid) (DTNB). As shown in Fig. 7A, 9,10-PQ modified GAPDH sulfhydryl groups anaerobically and the percent of thiols modified strongly correlated with inhibition of enzymatic activity ($r^2 \cong 0.90$). Linear regression analysis yielded slope of approximately unity. Similar results were also obtained for 1,4-BQ except that the slope obtained for this quinone was approximately twice that obtained with 9,10-PQ (Fig. 7B). As a homotetramer, GAPDH is known to exhibit different reactivities towards alkylating agents. The terms "half-of-the-sites reactivity" and "all-of-the-sites-reactivity" have been used to describe the complete loss of dehydrogenase activity when two or all four of the catalytic thiols have been modified, respectively [33,34]. This phenomenon has been reported with rabbit muscle GAPDH whose dehydrogenase activity exhibits all-of-the-sites reactivity towards *N*-(4-dimethylamino-3,5,-dinitrophenyl)-maleimide, but half-of-the-sites reactivity towards iodoacetamidonaphthol [35]. Furthermore, the dehydrogenase activity of yeast GAPDH has been extensively documented to exhibit half-of-the-sites reactivity towards chemically-diverse agents including iodoacetamide, 2-bromoacetamido 4-nitrophenol, and trifluoromethyl, acrylonitrile [33]. Stallcup and Koshland [33] have proposed ligand-induced negative cooperativity for the half-of-the-sites reactivity phenomenon, and Levitzki [34] has exclusively attributed such phenomenon to hydrophobic interactions of the alkylating agent with the adenine subsite region of the NAD+ binding site. When such interactions occur, conformational changes are transmitted to vacant subunits, resulting in half-of-the sites reactivity. All-of-the-sites reactivity, as observed with 9,10-PQ, results when such conformational changes are not transmitted. Thus, GAPDH exhibits different conformational changes after modification by 1,4-BQ and 9,10-PQ.

In an effort to further support the modification of GAPDH thiols by 9,10-PQ under anaerobic conditions, protection against inactivation by small thiols was assessed. Small thiols should scavenge 9,10-PQ and thus protect the enzyme. Thus, dialyzed GAPDH was incubated with 12.5 μM 9,10-PQ in the presence of increasing amounts of DTT. Following a 1-h anaerobic incubation, GAPDH activity was assayed to assess protection. As shown in Fig. 8, DTT protected GAPDH in a concentration-dependent manner. A similar trend was observed with other small thiols including GSH (data not shown). Notably, nearly complete protection against inactivation by 9,10-PQ was achieved with 100 μM DTT, which is 10-fold lower than the concentration that resulted in potent inhibition under aerobic conditions (Fig. 3A). These observations strongly suggest that the anaerobic inhibition of GAPDH by 9,10-PQ is mechanistically-distinct from that observed aerobically, and further supports the notion that this quinone inhibits GAPDH anaerobically through modification of thiols.

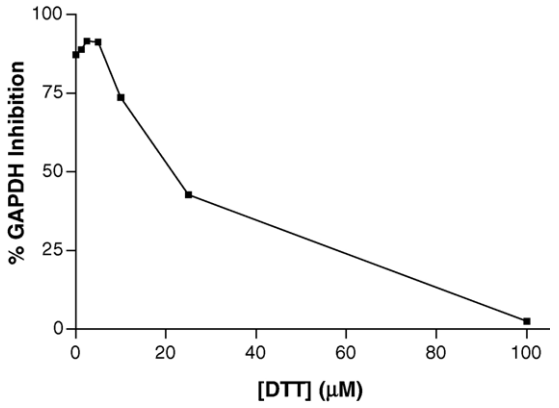

Fig. 8. Concentration-dependent protection by DTT against inactivation by 9,10-PQ under anaerobic conditions. Dialyzed GAPDH was incubated anaerobically with 12.50 μM 9,10-PQ for 1 h in the presence of increasing concentrations of DTT and the resulting GAPDH activity was measured. Values represent the average of duplicate determinations.

4. Discussion

In this study, we have examined the potential molecular mechanisms by which 9,10-PQ inactivates the glycolytic enzyme GAPDH. Incubations were carried out aerobically in the presence of DTT as a reducing agent to examine the role of ROS generation, and direct actions were examined by incubating dialyzed GAPDH with 9,10-PQ under anaerobic conditions. As an effective catalyst for reduction of oxygen by DTT, 9,10-PQ can generate ROS that could inactivate GAPDH by oxidizing its critical thiols. Consistent with this notion, the aerobic inactivation by this quinone was blocked by catalase. Furthermore, the aerobic inactivation by 9,10-PQ resembled that due to H_2O_2 in its partial reversal by excess DTT, which reduces protein mixed disulfides as a result of oxidation of GAPDH thiols to sulfenic acids. The observed aerobic inactivation rate constant of $0.078\,\mu M^{-1}\,min^{-1}$ is an excellent agreement with the reported value of $0.0618\,\mu M^{-1}\,min^{-1}$ for inactivation of protein tyrosine phosphatase CD45 by this quinone [36].

In addition to its aerobic effect, 9,10-PQ also inactivated GAPDH under anaerobic conditions in a manner that was consistent with irreversible inhibition, and comparable to the electrophilic actions of 1,4-BQ. Both quinones inactivated GAPDH at similar rates, but 1,4-BQ exhibited more than 10-fold greater affinity for the enzyme. Protection and thiol titration studies suggest that these quinones bind to the NAD+ binding site and modify GAPDH thiols from this site and not a nucleophilic residue, such as arginine. Indeed, the GAPDH active site has been reported as being a cleft between coenzyme binding and the catalytic domain, with the essential cysteine residue in close proximity to NAD+ [37]. The lack of protection by G-3-P was somewhat surprising since it forms a thiohemiacetal with the GAPDH active site thiol. However, in the proposed catalytic mechanism of GAPDH, NAD+ must bind before G-3-P, suggesting that NAD+-binding may be a required for G-3-P to interact with the GAPDH active site thiol [12,38]. Thus, the lack of protection by G-3-P may be attributed to the absence of NAD+.

The binding of 9,10-PQ to the NAD+ binding site is consistent with previous reports implicating the binding of this quinone to the NADPH binding site of neuronal nitric oxide synthase (nNOS) [39] and glutathione reductase (GR) [40,39]. The reported inhibition binding constants (K_i) were 1.8 and 0.38 μM for GR and nNOS, respectively. The higher K_i for 9,10-PQ and GAPDH may reflect the lower binding affinity of this quinone for NAD+ binding sites. The anaerobic modification of GAPDH thiols by 9,10-PQ to our knowledge is a new finding that may explain our previous report of anaerobic cellular toxicity by this quinone [11].

9,10-PQ is not likely to act as a Michael acceptor, but as an α,β-diketone, it contains two potential electrophilic carbonyl sites that are particularly susceptible to 1,2-addition to form thiohemiketals. Indeed, Schonberg et al. have reported the formation of unstable thiohemiketals by 9,10-PQ that partially decompose into their corresponding components [41]. Based on these arguments, it is possible that 9,10-PQ forms thiohemiketals with GAPDH thiols, which while reversible, may not readily dissociate because of binding of the rest of the quinone molecule. In other words, while GAPDH thiols provide the necessary reactivity, the binding of 9,10-PQ to the NAD+ binding site of GAPDH may impart specificity and stability. Initial attempts to characterize such adduct by electrospray mass spectrometry have been unsuccessful, possibly because of its relative instability.

The previously-observed anaerobic toxicity of 9,10-PQ to yeast cells [11] was unexpected as it lacks the typical α,β-unsaturation of Michael acceptors. However, the results of this study suggest that this quinone

may form a stable complex with the protein thiolate of GAPDH by directly binding to the NAD+ binding site. This interaction represents another mechanism by which 9,10-PQ can exert its toxic actions on tissue thiols.

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